LIPIDS IN HEALTHY AND BOTRYTIS - INFECTED BROAD BEAN LEAVES AND THEIR ROLE IN DISEASE DEVELOPMENT

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ABSTRACT

The lipids of three varieties of broad bean (Vicia faba L.) and the mycelium of the fungus Botrytis fabae sardina the cause of chocolate spot disease of beans were determined. The lipid pattern of 3-week-old leaves of the three bean varieties and the fungal mycelium were composed of phospholipids, free sterols, triglycerides, methyl esters of fatty acids and squalenes. Steryl esters of fatty acids were detected only in 6-week-old leaves of "Rebaia 40" and "Giza 3" bean varieties but not in "Giza 1" bean variety. The total lipids of the fungal mycelium were similarly devoid of the steryl esters of fatty acids. Phospholipid fatty acids 6-week-old healthy leaves of the three bean varieties are constituted of mono, di, and tri-unsaturated fatty acids. Poly-unsaturated fatty acids were not detected. Inoculation of bean seedlings with Botrytis fabae resulted in a marked decrease in most lipid groups. The role of steryl esters and lipids in general in the process of pathogenesis and disease resistance was discussed.

INTRODUCTION

It has been recently reported that lipids play a role in the process of pathogenesis and disease resistance (Hendrix 1970; Gottlieb, 1971, Hoppe and Heitefuss, 1974b & c, 1975 and Mondy & Koch, 1978).

Hoppe and Heitefuss (1975) determined the phospholipids and phospholipid fatty acids in healthy and rust-infected leaves of resistant and susceptiple bean varieties to Uromyces phaseoli. They found that infection of bean leaves resulted in the increase of phosphatidyl serine and a decrease in phosphatidyl glycerol content. At the same time, the phosphatidyl ethanolamine and phosphatidyl choline detected in the infected leaves contained higher amounts of unsaturated fatty acids than those from healthy leaves. Similarly, changes in other membrane lipids of Phaseolus vulgaris infected with Uromyces phaseoli were observed (Hoppe and Heitefuss, 1974 b & c).

On the other hand, lipids were recently reported to exert potent biological effects on fungi. They stimulate growth and many of the physiological activities of fungi and share in the structure and function of biological membranes (Hendrix, 1964, 1965; Wardle and Schisler, 1969; Lloyd et al., 1971 and Abu Shady, 1971 & 1979).

Therefore, it was of interest to determine the lipid pattern in healthy and Botrytis - infected broad bean leaves as well as in the mycelium of the fungus itself, aiming to throw some light on the role played by lipids in the process of pathogenesis.

MATERIAL AND METHODS

Three varieties of broad bean (Vicia faba L.) namely "Rebaia 40" (R_{40}) "Giza 3" (G_3) and "Giza 1" (G_1) were used in the present study. Seeds of these varieties were kindly provided by the Plant Breeding Department, Ministry of Agriculture, Giza, A.R. Egypt. Botrytis fabae sardina was isolated from broad bean leaves that showed typical symptoms of chocolate spot disease. Single spore culture of the fungus was prepared, and stored at 5°C for artificial infection of bean plant.

Artificial infection of four-week-old healthy broad bean seedlings was done by inoculation with Botrytis spore suspension. Spore suspension ($2X10^7$ spores/ml distilled water) was prepared from representative slants and sprayed by an atomizer. Seedlings of the three bean varieties were first sprayed with sterile water before the application of the fungal spore suspension. They were then kept under humid conditions in the green house. Characteristic chocolate spots were developed 10 days after inoculation. For each particular broad bean variety extraction and analysis of lipids were done from healthy leaves of 3-week-old seedlings as well as from infected and healthy leaves of 6-week-old seedling.

Extraction and analysis of lipids from bean leaves:

Total lipids of leaves were extracted by chloroform-methanol (2:1 v/v) and purified by washing with 0.7% saline solution (Folch et al.,1957). The lipids were analyzed by thin layer chromatography on silica gel G (E. Merck, Darmstadt, W. Germany). The plates were activated for 2-3 hours at 110°C prior to use. Total lipids were fractionated using the

solvent hexane-diethyl ether-acetic acid (90:10:1 v/v/v) (Mangold & Malins, 1960). For the fractionation of polar lipids the solvent system chloroform-methanol-water(65:25:4 v/v/v) was used (Wagner et al., 1961). To analyze the phospholipid fatty acids a direct methanolysis on the plate was carried out (Kaufmann et al., 1966). Two dimensional chromatograms were used to separate the phospholipid fatty acids from the total lipids. All processes of lipid extraction and analysis were carried out under carbon dioxide atmosphere. The lipid fractions were detected by spraying the plates with 5% phosphomolybdic acid in 95% ethanol and heating at 110°C, or by charring after spraying the plates with 50% aqueous sulphuric acid. The fractions were identified by comparing their migration rates to those of standard samples and by the reactions with specific spray reagents (Dittmer & Lester, 1964).

Extraction and analysis of lipids from Botrytis fabae mycelium:

For lipid extraction and analysis, stationary cultures of the fungus were grown in 500 ml Erlenmayer flasks containing 50 ml liquid medium with the following constitution per one litre tap water: glucose, 40 gm; peptone, 10 gm; potassium nitrate,0.1 gm; potassium dihydrogen phosphate, 6.8 gm; magnesium sulphate, 2.5 gm; calcium chloride, 0.1 gm; and ferric chloride, 20 mg. The medium was sterilized by autoclaving and inoculated with fungal spore suspension, then incubated at 21°C for two weeks. Flasks were then filtered and the mycelium was washed 3 times with distilled water, then dried under vacuum in CO_2 atmosphere and then subjected to lipid extraction and analysed as previously described.

Effect of individual lipid components of broad bean leaf extract on Botrytis spore germination:

Spores of Botrytis were allowed to germinate in different concentrations of lipids identified in the leaf extracts of the three broad bean varieties. The concentrations were 10,50,100 and 250 ppm. Freshly egg phospholipids were prepared according to (Folch et al.,1957) and directly emulsified in distilled water to make stock solution from which the proper concentrations were prepared.

Other lipids were first emulsified in tween 80 and then dissolved in distilled water. Controls

prepared were distilled water and distilled water containing tween 80. The percentage of spore germination in each treatment and controls was measured after 24 hours.

RESULTS

Lipid determinations:
a. Total lipids:

The total lipids of 3-week-old seedlings of the three broad bean varieties showed more or less the same pattern, with the exception of the presence of trace amount of methyl esters of fatty acids detected in the extract of "Giza 1" variety as compared with appreciable amounts of this lipid fraction detected in the extracts of "Rebaia 40" and "Giza 3" varieties (Table 1). The lipid pattern of the bean varieties was composed of phospholipids, free sterols, free fatty acids, triglycerides, methyl esters of fatty acids and squalenes.

A common observation is the absence of steryl esters of fatty acids from the lipid extract of the 3-week-old samples of the 3 broad bean varieties. The most striking result (Table 2) is the detection of steryl esters in the lipid extract from six-week-old "Rebaia 40" and "Giza 3" varieties.

Table 1. Identity and relative abundance of total lipids in extracts from 3-week-old "Rebaia 40", "Giza 3" and "Giza 1" broad bean varieties.

	Broad Bean Varieties		
Lipids	"Rebaia 40"	"Giza 3"	"GIza 1"
Phospholipids	++	++	++
Free sterols	++	++	++
Free fatty acids	+++	+++	+++
Triglycerides	+	+	+
Methyl esters of fatty acids	++	++	traces
Squalenes	++	++	++

The lipid extract of the 6-week-old leaves of "Giza 1" variety showed the absence of this fraction and the presence of traces of methyl esters of fatty acids. On the other hand, the results presented in (Table 2) indicate that the total lipid extracts of the noninoculated and inoculated bean leaves of the

Table 2. Identity and relative abundance of the total lipids in extracts from either inoculated or noninoculated leaves of 6-week-old bean varieties and those of Botrytis fabae mycelium.

Lipdis	Broad Bean Varieties						Botrytis mycelium
	"Rebaia 40"		"Giza 3"		"Giza 1"		
	(N)	(I)	(N)	(I)	(N)	(I)	
Phospholipids	++	++	++	++	++	++	+++
Free sterols	++	+	++	+	++	++	++
Free fatty acids	+++	+	+++	+	+++	+	+++
Triglycerides	+	+	+	+	+	+	+
Methyl esters of fatty acids	++	++	++	++	t	t	++
Steryl esters	+++	t	+++	t	–	–	–
Squalenes	++	++	++	++	++	++	+++

– absent ++ Present in appreciable amount
+ present +++ Present in high amount
t traces

three varieties (6-week-old) showed more or less the same lipid pattern detected in the extracts of leaves from 3-week-old plants. The total lipid content of the leaves is composed of phosholipids, free sterols, free fatty acids, triglycerides, methyl esters of fatty acids and squalenes.

Furthermore a marked quantitative change in the lipid patterns of non-inoculated and inoculated bean leaves has been observed. A general observation is the decrease of most lipid groups in the extracts of Botrytis-inoculated seedlings. In this respect, steryl esters were detected in appreciable amounts in the healthy "Rebaia 40" and "Giza 3" bean leaves in contrast to only traces detected in the inoculated seedlings. (Table 2).

Analysis of the total lipid extract of Botrytis fabae mycelium shows that lipid content of the mycelium is composed of phospholipids, free sterols, free fatty acids, triglycerides, methyl esters of fatty acids and squalenes. A striking result is the complete absence of steryl esters from the lipid extract of the fungus.

b. Phospholipid fatty acids:

Analysis of the lipid extracts of the three bean varieties (3-week-old) (Table 3) showed that the phospholipid fatty acids are composed of saturated,

mono-, and di-unsaturated fatty acids. Furthermore, traces of tri-unsaturated fatty acids (trienoic) were detected in the lipid extracts of "Rebaia 40" (R_{40}) and "Giza 3" (G_3) bean leaves. Polyunsaturated fatty acids were absent from the lipid extracts of the three broad bean varieties.

The phospholipid fatty acids of 6-week-old healthy bean leaves are composed of saturated, mono-,di-,and tri-unsaturated fatty acids. Polyunsaturated fatty acids were absent. Furthermore, it is evident (Table 4) that phospholipid fatty acids disappeared from the lipid extracts of the inoculated "Rebaia 40" and "Giza 3" bean leaves. Conversely neither qualitative nor quantitative changes were detected in the phospholipid fatty acids of inoculated and non-inocualted "Giza 1" bean leaves.

Table 3. Identity and relative abundance of phospholipid fatty acids in extracts from 3-week-old leaves of the three broad bean varieties.

Phospholipid fatty acids	Broad Bean Varieties "R 40"	"G 3"	"G 1"
Saturated	++	++	++
Monoenoic	+++	+++	++
Dienoic	+	+	+
Trienoic	traces	traces	–

Table 4. Identity and relative abundance of phospholipid fatty acids in extracts from either inoculated or non-inoculated leaves of 6-week-old bean varieties and those of Botrytis fabae mycelium.

Phospholipid fatty acids	Broad Bean Varieties "Rebaia 40" (N)	(I)	"Giza 3" (N)	(I)	"Giza 1" (N)	(I)	Botrytis mycelium
Saturated	+++	–	+++	–	+++	+++	++
Monoenoic	+++	–	+++	–	+++	+++	traces
Dienoic	++	–	++	–	++	++	–
Trienoic	+	–	+	–	+	+	–

– Absent ++ Present in appreciable amount
+ Present +++ Present in high amount

The phospholipid fatty acids of Botrytis mycelium contained only saturated fatty acids and traces of mono-unsaturated fatty acids (monoenoic) (Table 4).

Effects of lipid detected in broad bean leaf extracts on *Botrytis* spore germination:

The results presented in (Table 5) indicate that all the classes of lipids at all the tested concentrations stimulated spore germination of the fungus. However, steryl esters of fatty acids were superior in their stimulatory effect.

Table 5. Effect of lipids detected in broad bean leaf extracts on *Botrytis* spore germination.

Conc. of lipids (ppm)	Spore germination after 24 hr%			
	10	50	100	250
Lipids:				
Phospholipids	35	48	48	35
Free sterols	30	50	50	37
Free fatty acids	27	32	38	32
Triglycerides	30	35	35	35
Methyl esters of fatty acids	30	38	39	29
Steryl esters	28	55	68	45
Squalenes	31	35	38	30

Distilled water 25% (Control 1).
Tween 80 18% (Control II).
Data are means of 4 replicates, 10 microscopic field each.

DISCUSSION

Infection of broad bean leaves with *Botrytis fabae* resulted in several distinguishable alterations in the physiology of the host. The observed changes in lipids conform to the results reported for different host pathogen interactions.

The most interesting finding in the present work was the complete absence of steryl esters of fatty acids from the lipid extracts of all three broad bean varieties at the plant age of 3 weeks and from the fungal mycelium. On the other hand, steryl esters were detected in the leaf extracts of 6-week-old bean varieties, of "Rebaia 40" and "Giza 3" but not in "Giza 1" variety. Furthermore, the results indicate that the degree of susceptibility of the three broad bean varieties may be correlated in part to the presence of steryl esters in the leaf extracts of bean seedlings. In this respect it could be suggested that steryl esters of fatty acids may play a role in the infection and development of the chocolate spot

disease by Botrytis fabae. Also, it is worthy of mention caused here that the detection of steryl esters in the leaf extract of bean leaves may be correlated to their age. This suggestion explains why the older leaves were more susceptible than the younger ones and why the "Giza 1" variety was the least susceptible variety toward infection with Botrytis fabae.

Furthermore, steryl esters were superior in stimulating spore germination of Botrytis fabae. This finding supports the above suggestion that Steryl esters may play a role in the infection development of chocolate spot disease. The absence of steryl esters from the lipid extract of the fungus Botrytis fabae and the presence of traces of this lipid fraction in the lipid extract of infected broad bean leaves of "Rebaia 40" and "Giza 3" varieties compared with large amounts of steryl esters detected in non-infected ones is a result which indicates that steryl esters were completely utilized by the fungus. This finding confirms that steryl esters may play a role in disease development.

Concerning the effect of other lipid groups on spore germination of the fungus detected in leaf extracts, phospholipids and free sterols, free fatty acids and triglycerides, squalenes and methyl esters of fatty acids could be arranged in a descending order with respect to their stimulatory effect. In this respect, it was reported that lipids stimulate the spore germination of fungi and have a role in the sporulation and physiological aging of some micro-organisms (Hendrix, 1964 & 1965; Haskins et al., 1964; Leal et al., 1964; Chee & Turner 1965; Lloyd et al., 1971). Growth of fungi was also stimulated by lipids (Wardle and Schisler, 1969 and Smith 1970).

On the other hand, the decrease of lipids detected in the two infected varieties of Vicia faba seedlings namely, "Rebaia 40" and "Giza 3", could be attributed to the enhanced degradation of plant cell membranes. An increased membrane degradation in the infected tissue might be of great importance for cell permeability which undoubtedly plays a part in the nutrient supply available to the invading pathogen (Hoppe and Heitefuss, 1974a). In this respect, it is known that membrane lipids can determine the barrier properties of a membrane (Van Deenen 1969). Accordingly, the absence of steryl esters from leaves of the variety "Giza 1" could explain its resistance.

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